Mosquito Surveillance Program Using Ovitraps Detected *Aedes aegypti* at the Honolulu International Airport in 2012

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Abstract. A mosquito surveillance program using ovitraps at the Honolulu International Airport (HIA), Hawaii, USA May 2010 to June 2012 revealed that *A. albopictus* egg counts fluctuated over time during the surveillance program and the highest oviposition was observed from February to May 2011 and the lowest was from September to November 2011. Positive correlations found between a given week's rainfall and egg counts 1 and 2 weeks later suggested that rainfall triggered the hatching of eggs which were laid before the rainfall, rather than directly stimulating adult oviposition. In January and June 2012, *Aedes aegypti* was discovered from a small vegetated area located between terminals for out-of-state and within-state flights. This species had not been confirmed present on Oahu at least since 1948. This finding has led us to intensify our surveillance program at the airport, with the hope that we would improve our understanding of the nature of mosquito introductions at this important port of entry for the Hawaiian Islands.

Key words: *Aedes albopictus*, *Aedes aegypti*, ovitrap, invasive pest surveillance

The Asian tiger mosquito, *Aedes albopictus* (Skuse), and the yellow fever mosquito, *Aedes aegypti* (Linnaeus), were introduced into the main Hawaiian islands in the 1890s. Both species became established in Hawaii and were important in several dengue outbreaks in Hawaii until the 1940s (Usinger 1944, Gilbertson and Engineer 1945). However, as a consequence of the dengue outbreak of 1943–44, a successful mosquito control program eradicated *A. aegypti* from the islands of Kauai and Oahu, as shown by a statewide survey in 1948 (Hess 1957). At that time, *A. albopictus* remained established on the islands of Kauai, Oahu, Maui, Molokai, Lanai, and Hawaii while *A. aegypti* remained on Maui, Molokai, and Hawaii (Hess 1957).

In 2002, staff of the Vector Control program of the Hawaii Department of Health conducted a statewide survey for these two mosquito species in response to a 2001–2002 dengue outbreak. *A. albopictus* was collected from all surveyed islands, Kauai, Oahu, Maui, Molokai, Lanai, and Hawaii, but *A. aegypti* was captured only from isolated locations on Hawaii island (Effler et al. 2005). Consequently, the last occurrence of an established population of *A. aegypti* on the island of Oahu was from the period 1944 to 1945 (Bonnet 1947, Hess 1957).

Currently, no mosquito-borne human diseases are endemic to Hawaii. However, there is a chance that viruses

such as chikungunya and dengue could become established within the state. There are approximately six confirmed dengue cases imported to Hawaii each year (Department of Health 2015), and chikungunya was imported for the first time in 2014 (Centers for Disease Control and Prevention 2015). Imported cases like these are typically unconnected and consist of illness beginning in Hawaii residents just before, or soon after, return from international travel. Sporadic cases of humans harboring dengue infection in Hawaii apparently do not lead to outbreaks of illness due to the relatively limited vectoring competency of *A. albopictus*. Even so, a surveillance program for *A. albopictus* is very important to timely detection and control of any unusual increase in abundance of this dengue vector in the state. *A. albopictus* was implicated in autochthonous transmission of dengue virus infection in Hawaii in 2001–2002, in conjunction with a spatial and temporal cluster of travelers carrying infection. Reestablishment of *A. aegypti* in densely populated areas of Oahu would markedly change the disease vectoring potential within the state. For these reasons combined, *Aedes* mosquito species merit special public health attention.

For decades previous to this project, gravid and light traps were standardized components of public health mosquito surveillance throughout the state, including at the Honolulu International Airport (HIA). Those methods were terminated in November 2009 in conjunction with funding and staffing reductions for the State of Hawaii Department of Health. In recent years, rapid spread of *Aedes* mosquito species from native ranges or established areas of introduction has been attributed to international commercial exchanges and international travel (Fonseca et al. 2001, Schaffner et al. 2004, da Costa-da-Silva et al. 2005, Brown et al. 2011). These observations lend credence to the ongoing importance of public health surveillance in Hawaii at likely points of vector introductions and establishments. Following program reorganization, mosquito surveillance was resumed only at the HIA. This paper reports on mosquito surveillance data collected at HIA from May 2010 to June 2012 using ovitraps. The primary objectives of this surveillance effort were (1) to monitor the long-established *A. albopictus* around the HIA and (2) to survey for the introduction of mosquito species new to Oahu or the state. We report here collections of *A. aegypti* at HIA after a 64 to 67-year absence of breeding on Oahu. We also present parallel mtDNA haplotype data for these collections and from a sample of the nearest known *A. aegypti* population, a long-established, isolated population on Hawaii island, approximately 300 km away from Oahu. Data are also presented on the association between rainfall at HIA and collections of *A. albopictus* and *A. aegypti* eggs from ovitraps.

Materials and Methods

Mosquito collection sites and rainfall data. Twenty ovitraps were placed at four sites (five traps each) around HIA (Figure 1) for two years from May 2010 to June 2012. The collection period consisted of 111 weeks in total. At HIA, northeast and east trade winds occurred 210 days per year in 2009 (Garza et al. 2012). Sites 1 and 4 get trade winds more directly than Sites 2 and 3 because the latter are surrounded by vegetation and buildings. Ovitraps were set up with 2-day-old infusions of grass and tap water (20 g of dry grass in 8 L of water). Traps were replenished with infusions once per week. Mosquito eggs were collected once per week by collecting wooden paddles from the ovitraps and drying them for 10 days. All eggs, including live eggs, egg shells, and dented eggs,

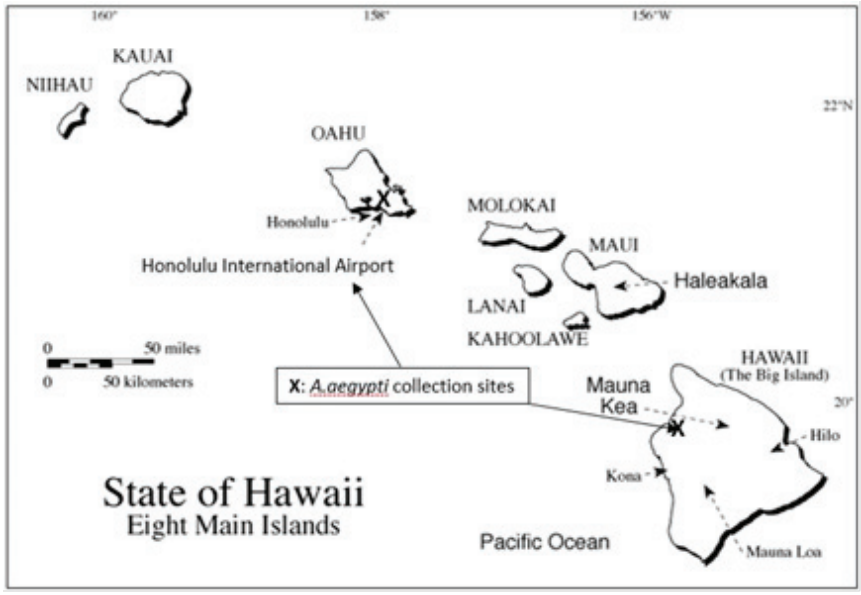

Figure 1. Map of the Hawaiian Islands. Markers of "X" represent each of this study's *Aedes aegypti* collection sites on Oahu and Hawaii islands.

were counted. Live mosquito larvae and pupae were observed in the infused water of the ovijars, and eggs were observed on the walls of the ovijars. We collected all larvae and pupae from traps for species identification; however, we did not include these in total egg counts because there was uncertainty whether immature mosquitoes from the ovijars were from egg shells observed on paddles or from the walls. The numbers of immature mosquitoes from ovijar water were typically < 10 % of the total counted eggs from paddles. All live eggs were reared under lab conditions for species identification.

Laval stages of *A. aegypti* were collected from Hawaii island (Figure 1) and reared to adults in the lab. These specimens used for genetic analyses.

Weekly rainfall data were obtained from Wunderground.com (2015).

Variation among collection sites in egg collections. A Friedman test was applied using the "friedman.test" function in R version 3.1.1 (R Core Team 2014) to counts of egg collections, grouped by collection week and blocked by collection site, to test whether the distributions of egg counts were equal among sites.

Correlation between rainfall and egg collections. Mosquito breeding is influenced by rainfall (Gilberson 1945). Standing water is needed for oviposition and larval development of *Aedes* species while rainfall is required for egg hatching in outdoor oviposition sites like small containers (Soti et al. 2012). To test for positive or negative associations between weekly rainfall totals and egg collections, two-sided Spearman rank correlation (*rho*) was used. Correlation tests were performed using the "cor.test" function in R version 3.1.1 (R Core Team 2014). To investigate the possibility of time lags between rainfall and egg collection, correlation tests were conducted with no time lag (i.e., each week's total rainfall compared with the same week's total egg

collections) and with time lags of 1 to 5 weeks (e.g. each week's rainfall compared with egg collections 1 week later). Correlation coefficient values were rounded to two decimal places. Per-test statistical significance was calculated by the default, exact algorithm, AS 89 (Best and Roberts 1975), and is reported as *p*-values rounded to three decimal places.

Mitochondrial gene amplification. To investigate the origin of collected *A. aegypti* mosquitoes, we conducted partial amplification of two mitochondrial genes using PCR. Dried adult males and females of *A. aegypti* were preserved at –20°C until DNA extraction. DNA extraction was conducted at the Department of Health State Laboratories main facility. DNA was extracted from two legs (January 2012 collected *A. aegypti* at HIA) or whole bodies (June 2012 collected *A. aegypti* at HIA and Hawaii island) of individual mosquitoes using the DNeasy animal blood and tissue kit (QiaGen). We amplified a 359-basepair (bp) region of the NADH dehydrogenase subunit 4 (ND4) gene using published primers (da Costa-da-Silva et al. 2005) and a 580-bp region of the *cytochrom oxidase* subunit I (COI) gene using published primers (Beebe et al. 2005). PCR reactions were carried out in 25 μl volume consisting of 4 μl of extracted template DNA, 3 μl of $MgCl_2$ (25 mM), 1 μl of dNTPs, 2.5 μl of 10X PCR buffer, 1 μl of forward and reverse primer each, 0.18 μl of Taq polymerase and 11.32 μl of water, using program emulation 9700 on Variti Themal Cycler (Life Technolgies, Applied Biosystem, Grand Island, NY). All runs included a negative control (water substituted for template). The PCR program consisted of an initial incubation at 95°C for 3 minutes; 40 cycles at 95°C for 1 minute, 45°C (COI) or 48°C (ND4) for 1 minute, and 72°C for 1 minute; followed by 72°C for 10 minutes. PCR products were stored at –20°C. The sizes of PCR amplicons were confirmed by agarose gel electrophoresis. There was no amplicon in negative controls for all the PCR runs. PCR amplicons were cleaned using a commercial purification kit (Qiagen) and sequenced at the University of Hawaii at Manoa, Advanced Studies in Genomics, Proteomics and Bioinformation. Original forward and reverse PCR primers were used for sequencing. The sequences were compared with sequences available from GenBank using BioEdit (Ibis Biosciences, Carlsbad, CA). The sequences were deposited in GenBank under accession numbers KU207145 (COI haplotype) and KU207146 (ND4).

Phylogenetic analysis of ND4 and COI haplotypes. Phylogenetic relationships of Hawaiian haplotypes of ND4 and COI were estimated using PAUP 4b10 (Swofford D.L. 2001) with maximum parsimony and distance/ neighbor joining analyses. Branch support in the phylogeny was estimated by bootstrap analyses of 1,000 replicates. ND4 sequence of *A. albopictus* collected from HIA was designated as an outgroup.

Results

***Aedes albopictus* egg collection by ovitraps.** Among all collected *A. albopictus* eggs (n=13347), 83% (n=11079) were live eggs and 17% (n=2197) were either eggs shells or dented eggs. Mean numbers of eggs collected from all 4 sites are presented in Figure 2. The frequency distributions of egg collections per week and the weekly abundances of egg collections differed across sites (Friedman $\chi^2 = 174.05$, df = 110, $p < 0.001$). Site 1 and Site 4 had no eggs collected in 68-74% of weeks while Site 2 and Site 3 had no eggs collected in 42% and 14% of weeks, respectively (Figure3). Heterogeneity in the collection results suggested that the four separate collection sites at the airport represent different environments for oviposition by

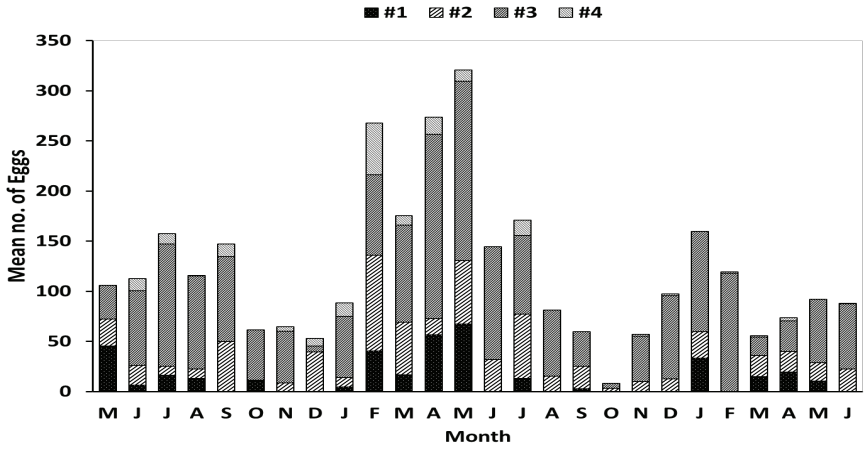

Figure 2. Mean number of eggs collected monthly using ovitraps from Honolulu International Airport from May 2010 to June 2012. Monthly values are the averages of collections for all weeks in the month, by collection site.

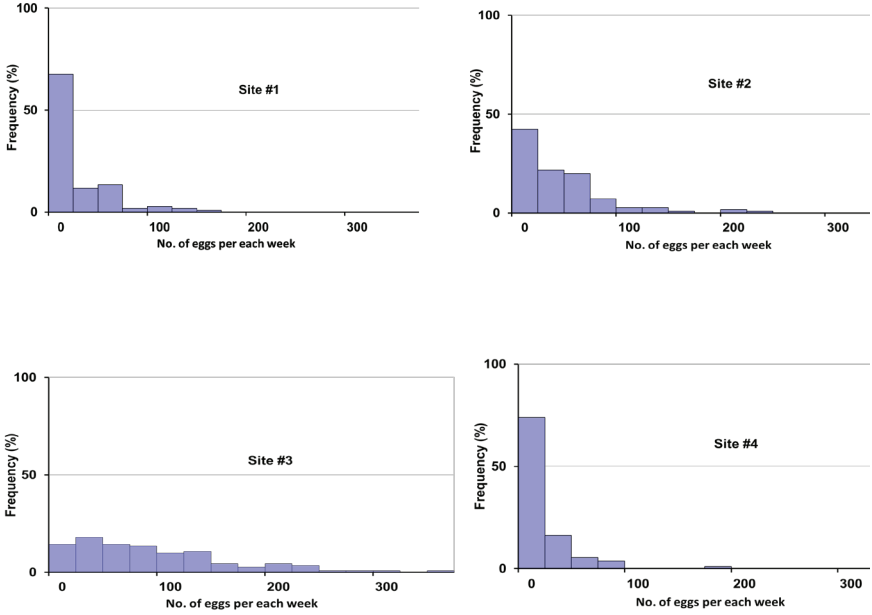

Figure 3. Frequency distribution of egg collections for each ovitrap site at Honolulu International Airport

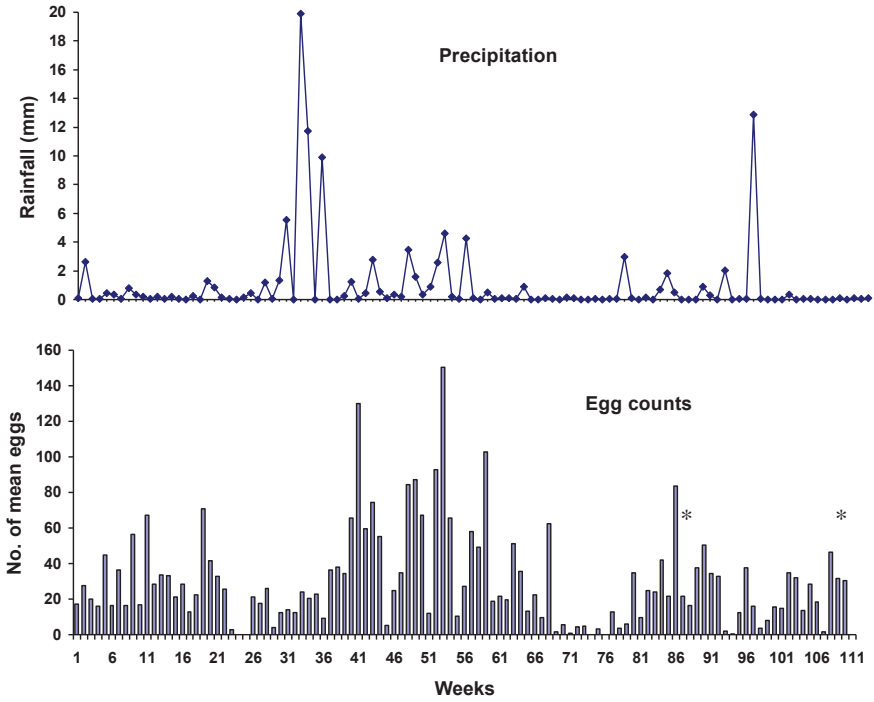

Figure 4. Mean number of eggs collected per week using ovitraps and mean rainfall per week at Honolulu International Airport from May 2010 to June 2012. * shows the weeks (87 and 110) in which *A. aegypti* was collected.

mosquitoes. However, seasonal oviposion patterns were similar at all locations. The highest oviposition was observed from February to May 2011 and the lowest egg counts were observed from September to November 2011 (Figure 2, 4).

***A. aegypti* egg collection by ovitraps.** *A. aegypti* was detected January 9–17, 2012 (week 87) (Figure 4) from Site 3, a small vegetation area along the bridge way located between terminals for out-of-state and within-state flights. Twenty-one eggs were collected then at that site from one of the five ovitraps. Eight females and five males of *A. albopictus* and four females and four males of *A. aegypti* were identified after rearing to maturity.

One hundred fourteen eggs were collected on June 18–25, 2012 (week 110) (Figure 4) from the same ovitrap which collected *A. aegypti* the previous January, and all 96 live eggs were matured and identified as *A. aegypti* (45 female, 51 male). No *A. albopictus* was identified in this collection.

Correlation between rainfall and egg collections. There was no significant correlation between rainfall and total egg counts within weeks ($rho = 0.16$, $p = 0.101$). The only significant correlations seen using time-lagged data were positive correlations between a given week's rainfall and egg counts 1 week ($rho = 0.30$, $p = 0.001$) and 2 weeks ($rho = 0.26$, $p = 0.006$) later. These two correlations remain significant under a Bonferroni correction for multiple testings, whereby the adjusted significance threshold would

be 0.05 divided by 6 = 0.008.

Mitochondrial DNA haplotypes and phylogentic relationships of *A. aegypti* collected from Honolulu International Airport and Hawaii island. Only one haplotype each of ND4 and COI genes was found from *A. aegypti* mosquitoes collected in January (n=8) and June (n=10) 2012 at HIA and collected in August (n=34) 2012 from Hawaii island in this study: there was no genetic variation between the HIA specimens and those from Hawaii island. Phylogenetic analysis showed that these Hawaii haplotypes are closely related to ones found in southeast Asian countries (Figure 5).

Discussion

The *A. aegypti* collections reported here constitute the first confirmation of this species on the island of Oahu since the eradication report of *A. aegypti* on Oahu in 1948 (Hess 1957). This study presents limited genetic information on a geographically-restricted population of *A. aegypti* from Hawaii island and these recent, Oahu collections. The Hawaii island population is believed to have continuous occurrence there since the 1890s. No genetic variation was detected among *A. aegypti* mosquitoes from Hawaii island (n=34) and from HIA (n=18) in this study, by the gene regions amplified from individual specimens.

The HIA collections were from one of four surveillance locations, in a vegetated area between out-of-state and within-state terminals. *A. aegypti* were collected twice during this 111-week study from May 2010 to June 2012, at weeks 87 and 110. Both sets of *A. aegypti* collections were obtained from the same ovitrap (one of five) at the site. This site-specific repetition of *A. aegypti* collections—with no *A. aegypti* collections but ongoing *A. albopictus* collections in that ovitrap during the intervening weeks—indicates potentially important roles for small-scale environmental differences and for the physical location of that site, for intercepting *A. aegypti* by ovitraps.

Larger-scale seasonal patterns in *Aedes* mosquito oviposition were also observed. There are two seasons in Hawaii: a warmer, dry season from May to October and a cooler, rainy season from November to April (University of Hawaii Department of Geography 1983). While both the lowest and highest oviposition were observed in the warmer, dry season (respectively, September–October in 2010 and 2011; and March and June 2011), most of the seasonal increase in mosquito oviposition after the end of the dry season occurred in the 2nd to 3rd months (December and January) of the rainy season. This is similar to what was reported by Gilbertson and Engineer (1945): increasing rainfall in early December interrupted the decreasing *Aedes* mosquito breeding index and when the annual rainy season developed in February and March mosquito breeding more than tripled in one month.

This observed seasonal oviposition pattern is being used in the continuing mosquito surveillance program around the airport. Vigorous searching for breeding sources occurs not only in the rainy season but also in the dry season when oviposition can peak. During the course of this study, in work separate of that reported here, when the egg count of *A. albopictus* was unusually high (Figure 4: week 39 and 51), we were able to identify previously unidentified breeding sources in the vicinity of the airport by intensive searching effort due to the population spike.

Our study showed that a decrease of mosquito oviposition from late September to October was interrupted by rainfall in middle November and early December. Rainfall is known to trigger egg hatching and affect adult production of *Aedes*

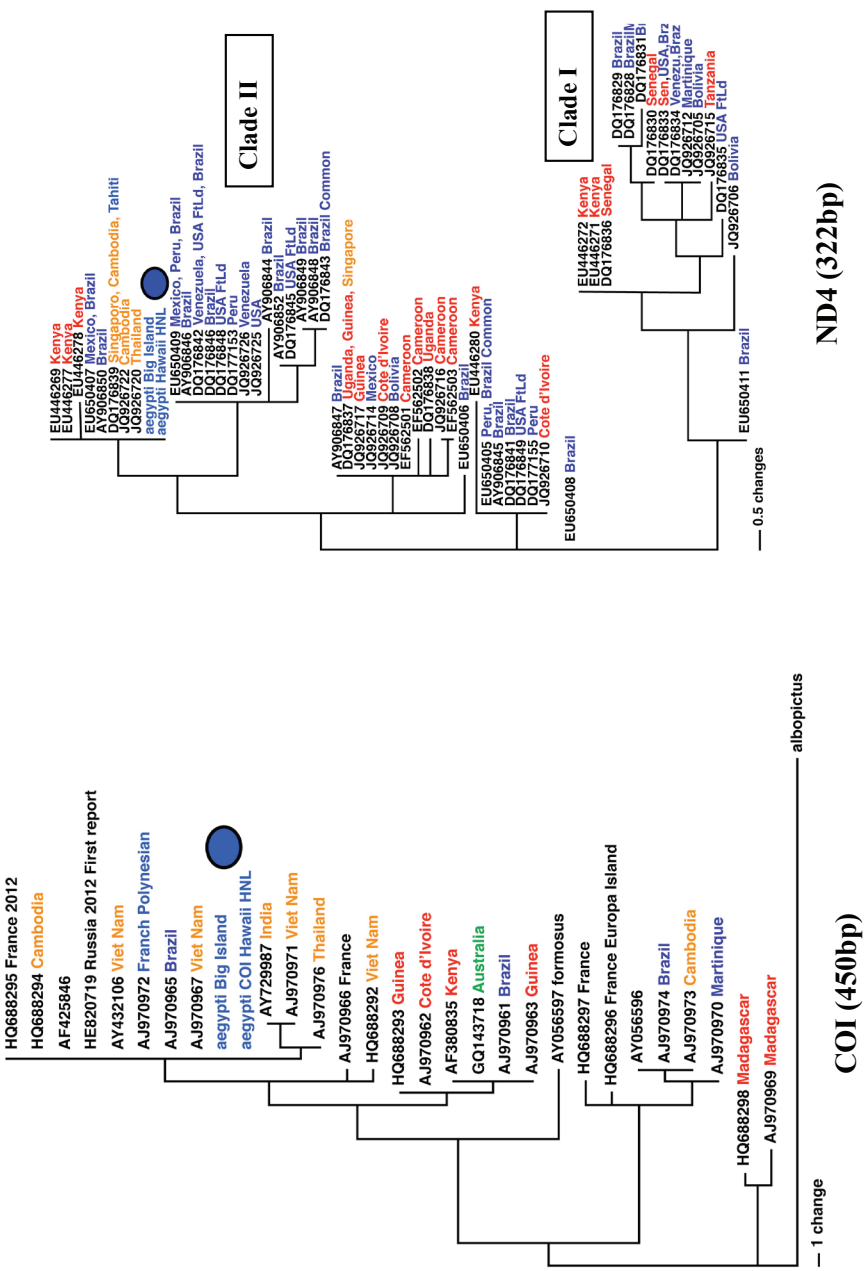

Figure 5. Neighbor-joining tree for *A. aegypti* based on COI (450bp) and ND4 (322bp) sequences. Labels are Genbank accession numbers combined with country names.

species breeding in outdoor oviposition sites (e.g., Alto and Julian 2001, Soti et. al. 2012). Observational and experimental studies have shown that the relationship between *A. albopictus* oviposition and rainfall is complex, especially in urban environments (e.g., Alto and Juliano 2001, Waldock et al. 2013, and references therein), with some studies finding no correlation between rainfall and breeding activity. A positive correlation was found in this study between a given week's rainfall and egg counts 1 and 2 weeks later, indicating that rainfall immediately triggered egg hatching and subsequently effected greater adult production, both increasing egg counts. Based on independent field observations from other locales below 300 meters elevation in Hawaii (data not shown), after eggs hatch, maturation to the adult stage generally takes 7–10 days.

Previous studies have identified two distinct genetic lineages of both ND4 and COI mitochondrial genes for *A. aegypti* (Bosio et al. 2005, Bracco et al. 2007, Sacarpassa et al. 2008, Lima and Scarpassa 2009). Clade I has been identified from African and American specimens. Clade II has been identified in African and Asian specimens. The haplotypes recovered in this study clustered within Clade II (Figure 5).

The ND4 haplotype recovered in this study is also known from Thailand (H5 of Bosio et al. 2005), Brazil (H3 of Lima and Scarpassa 2009), Mexico (H5 of Gorrochotegui-Escalante et al. 2002), Asia (H13 of Bracco et al. 2007), Venezuela (HMex5 of Urdaneta-Marquez et al. 2008) and South and North American countries (H12 of Lima and Scarpassa 2009). Those American haplotypes have been discussed as secondary introductions to the Americas during 1980s as a consequence of the intense commercial exchange with Asian countries and commercial globalization (Bracco et al. 2007).

The COI haplotype recovered in this study is also known from France, Cambodia, Russia, Vietnam, Brazil, Thailand, and French Polynesia (Mousson et al. 2005, Sacarpassa et al. 2008, Beebe et al. 2005). This haplotype is one mutational step (1 nucleotide) from a widely dispersed Australian haplotype (H1, Beebe et al. 2005) and a Bolivian haplotype (H8, Paupy et al. 2012) of limited geographic distribution. Bolivian H8 was discussed as an escaped group from the DDT-based eradication programs reportedly completed in 1943 in Bolivia. The low prevalence of this lineage and its ecological restriction to rural settings—perhaps a secondary adaptation—supports this possibility.

Based on phylogenetic analyses of haplotypes from mitochondrial COI and ND4 genes, *A. aegypti* collected from HIA in January and June 2012 were genetically similar if not identical to distant Asian populations and a geographically much closer, isolated, within-Hawaii population from about 300 km away.

There are many unaddressed aspects to the question of the most-likely source of the introduction(s) of *A. aegypti* reported in this paper. The severe limitations of the data currently available—including the complete lack of recent surveillance data from places other than HIA—mean that there is little basis for discriminating among competing hypotheses. For now, we believe it is reasonable to say that initial review of the limited information available is consistent with a hypothesis that the *A. aegypti* collected in January and June of 2012 at HIA were the result of mosquitoes transported to that vicinity by air travel in the prior 1–2 weeks, with the source of the mosquitoes being relatively nearby Hawaii island. However, other explanations cannot be ruled-out.

In conclusion, the potential establishment of *A. aegypti* on Oahu raises serious

concerns about the possibility of local arbovirus transmissions of pathogens such as dengue, chikungunya, and zika viruses. HIA has already been recognized as having one of the highest potentials to be the focus of an epidemic due to the volume of travelers from Pacific island and southeast Asian locales, where vector borne diseases are endemic. HIA is a destination and a way station for recreation and commerce between the Americas, Australia, and Asia. Hawaii also has a very active military presence which extends its reach world-wide. This recent finding of *A. aegypti* has led us to intensify our surveillance program at HIA, with the hope that we will improve our understanding of the nature of mosquito introductions at this important port of entry for the Hawaiian Islands. For example, recent efforts led to a report on the first known collection of *A. japonicus* on Oahu (Yang and Hasty 2013). Still more effort is needed; we must continue active surveillance for mosquito introductions in Hawaii.

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